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Evidence for a Two-Step Mechanism Involved in Assembly of Functional Signal Recognition Particle Receptor

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Abstract. The signal recognition particle (SRP) and SRP receptor act sequentially to target nascent secretory proteins to the membrane of the ER. The SRP receptor consists of two subunits, SRα and SRβ, both tightly associated with the ER membrane. To examine the biogenesis of the SRP receptor we have developed a cell-free assay system that reconstitutes SRα membrane assembly and permits both anchoring and functional properties to be assayed independently. Our experiments reveal a mechanism involving at least two distinct steps, targeting to the ER and anchoring of the targeted molecule on the cytoplasmic face of the membrane. Both steps can be reconstituted in vitro to restore translocation activity to ER microsomes inactivated by alkylation with *N*-ethyl-maleimide. The characteristics elucidated for this pathway distinguish it from SRP-dependent targeting of secretory proteins, SRP-independent ER translocation of proteins such as prepromellitin, and direct insertion mechanisms of the type exemplified by cytochrome b5.

THE initial step in sorting proteins to a number of intracellular organelles as well as to the exterior of the cell involves targeting the nascent polypeptide to the ER membrane. Cell-free systems in which this targeting event is reconstituted have allowed the identification of three distinct pathways used to direct proteins to the ER membrane of higher eukaryotes (reviewed in Perara and Lingappa, 1988; Sabatini et al., 1982). Two of these pathways involve recognition of a signal sequence encoded in the nascent protein by specific receptors in the cytoplasm and on the ER membrane. These two pathways differ in that one pathway is dependent on a cytoplasmic adaptor called signal recognition particle (SRP).[1] This SRP-dependent targeting pathway (reviewed in Walter et al., 1984) appears to be the primary route of targeting of both secreted and integral membrane proteins to the ER. The other pathway is independent of SRP, appears to be receptor mediated, and is responsible for targeting a few highly specialized small proteins such as prepromellitin (Muller and Zimmermann, 1987). The third pathway, typified by cytochrome b5, uses a receptor-independent hydrophobic insertion sequence instead of a receptor-mediated signal sequence. Such sequences have been identified for only a small number of molecules and tend to be located at the extreme carboxyl terminus of the protein anchoring them to the cytoplasmic face of the membrane (Sabatini et al., 1982).

Two essential components of the SRP-mediated ER targeting system, SRP and SRP receptor (also called docking protein) have been isolated and characterized in some detail (Siegel and Walter, 1988; Tajima et al., 1986; Meyer et al., 1982). These two macromolecular complexes act sequentially to direct ribosomes synthesizing secreted and transmembrane proteins to the ER membrane. The process begins with the emergence of a signal sequence from the ribosome. SRP binds both the signal sequence and the ribosome directly (Krieg et al., 1986; Kurzchalia et al., 1986). This interaction slows elongation of the nascent polypeptide and maintains the nascent-chain ribosome complex in a translocation competent state. This complex is presumed to target to the ER membrane due to the affinity of SRP for its receptor on the cytoplasmic face of the ER (Gilmore et al., 1982*b*). After interaction with the SRP receptor, SRP loses its affinity for the signal-bearing translation complex and releases the now targeted nascent protein to the translocation machinery in the ER membrane (Gilmore and Blobel, 1983).

The SRP receptor is composed of two subunits, termed SRα and SRβ, which cofractionate in sucrose gradients and can be coprecipitated using antisera directed against either molecule (Tajima et al., 1986). The cDNA of the larger of the two subunits SRα, has been cloned from both canine (Lauffer et al., 1985) and human (Hortsch et al., 1988) cells. SRα has a deduced molecular mass of ~69,700 D and migrates in SDS-PAGE with an apparent molecular mass of ~72,000 D. It is this subunit that is believed to interact with SRP to release translation arrest and initiate translocation (Gilmore et al., 1982*a*).

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1. *Abbreviations used in this paper*: EF, elastase fragment; NEM, *N*-ethylmaleimide; SRα, signal recognition particle receptor alpha subunit; SRβ, signal recognition particle receptor beta subunit, SRP, signal recognition particle.

SRα has a large cytoplasmically disposed domain that can be cleaved from the membrane by low concentrations of either trypsin or elastase (Gilmore et al., 1982*a*; Meyer and Dobberstein, 1980). Amino terminal sequencing of this domain was used to confirm that it corresponds to a fragment of the molecule beginning at amino acid 152 and continuing to the end of the molecule, amino acid 638 (Lauffer et al., 1985). This cytoplasmic fragment has been shown to have a greatly reduced affinity for SRP suggesting that part of the molecule which remains membrane associated contributes to a functionally important domain of the receptor (Lauffer et al., 1985).

Microsomes treated with either trypsin or elastase to remove SRα are not functional in promoting nascent preprotein translocation or in releasing the SRP-induced arrest of translation observed in wheat germ translation reactions (Gilmore et al., 1982*a*). However both functions can be restored by adding back purified cytoplasmic fragment of SRα to the digested microsomes (Gilmore et al., 1982*b*).

Examination of the deduced amino acid sequence for SRα from a canine cDNA clone revealed several remarkable features (Lauffer et al., 1985) also found in the human sequence (Hortsch et al., 1988). At the amino terminus of the molecule are two hydrophobic sequences, amino acids 1–22 and 64–79, believed to anchor the molecule on the ER membrane. After the hydrophobic sequences are three highly charged stretches of amino acids, including residues 84–97, 129–175, and 205–243. The primary elastase cleavage site, between residues 151 and 152, is approximately midway through the second highly charged region of SRα.

SRα molecules synthesized in wheat germ cell-free translation reactions and then added to microsomes were observed to cofractionate with the membranes even after disruption of the microsomes with sodium carbonate. These experiments have been used to suggest the molecule may associate with microsomes posttranslationally (Hortsch and Meyer, 1988). However, the lack of a functional assay combined with the difficulties involved in controlling for nonspecific association in such an assay has made interpretation of the data difficult.

To examine the biogenesis of the SRP receptor we have developed a cell-free system that permits the introduction of recombinant molecules of SRα. In this assay both targeting and functional properties of the introduced molecules can be assayed independently. Here we show that SRα can be specifically recruited to functional sites on the ER membrane in vitro. Furthermore, targeting and anchoring are independent, separable steps. Targeting is saturable and can occur posttranslationally. SRα molecules target to microsomes treated with either trypsin or the alkylating agent *N*-ethylmaleimide (NEM). Stable anchoring in the membrane is also NEM resistant but can be abolished by pretreatment of microsomes with trypsin. Furthermore we confirm that amino terminal sequences removed from the full length molecule by elastase are required to achieve a stable association with the ER membrane. Together these results suggest SRα molecules are targeted to the ER membrane by a novel mechanism involving an as yet unidentified protein intermediary.

Materials and Methods

General Methods

Restriction endonucleases were obtained from Boehringer Mannheim Diagnostics, Inc. (Houston, TX) or from New England BioLabs (Beverly, MA) and were used according to the manufacturers instructions. Placental RNAse inhibitor was from Promega Biotec (Madison, WI). Rabbit anti-ovine prolactin was from United States Biochemical Corp. (Cleveland, OH). Preparation and characterization of the monoclonal antibodies directed against SRα and SRβ have been described (Tajima et al., 1986). Proteinase K was from E. Merck (Darmstadt, FRG), [^{35}S]methionine translabel from ICN Biomedicals, Inc. (Costa Mesa, CA), and Triton X-100 from Boehringer Mannheim Biochemicals (Indianapolis, IN). A mitochondria-enriched vesicle fraction was prepared from canine pancreas as described (Greenwalt, 1974), except that tissue homogenization and the initial centrifugation step employed a buffer containing 50 mM TEA, pH 7.5, 0.25 M Sucrose, 50 mM KoAc, 6 mM MgAc, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF.

Canine pancreatic microsomal membranes were prepared as described (Walter and Blobel, 1983). All microsome fractions were washed twice with 0.5 M KoAc to remove endogenous SRP and then EDTA stripped as described (Walter and Blobel, 1983). Trypsinized and NEM-treated microsomes were prepared as described (Gilmore et al., 1982*a*). Unless indicated the concentrations of trypsin and NEM used were 5 μg/ml and 2 mM, respectively. Inactivated microsomes were prepared in 100-μl aliquots at a concentration of five equivalents per microliter and were pelleted by centrifugation at ~110,000 *g* (20 psi for 10 min in an air-fuge [rotor A-100/30; Beckman Instruments, Inc., Fullerton, CA]).

Recombinant DNA Constructs

The complete coding sequence of SRα was assembled from recombinant phage clones λSR31 and λSR50 using a unique Nco I site at nucleotide position 1,546 of the cDNA sequence (Lauffer et al., 1985). To facilitate further

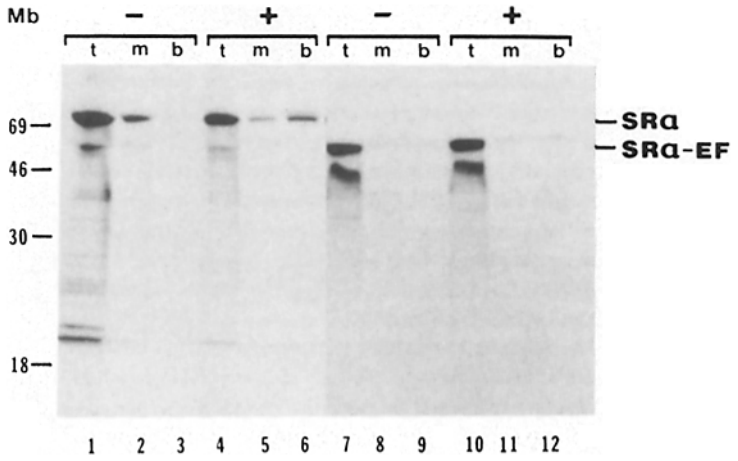

Figure 1. Cofractionation of SRα but not SRα–EF with microsomes. Reticulocyte lysate translation products of SRα (lanes *1–6*) and SRα–EF (lanes *7–12*) were synthesized in the presence or absence of added microsomes as indicated by *Mb*. Reactions were adjusted to 2 M urea, layered on sucrose step gradients, and centrifuged for 15 min at 30 psi (178,000 *g*). Gradients were divided into top, middle, and bottom fractions, indicated above the lanes as *t*, *m*, and *b*, respectively. Migration positions of molecular mass markers, in kilodaltons, and of SRα and SRα–EF are indicated on the sides.

manipulation the plasmid pSPSR9 was generated from nucleotides 1 to 2,555 of the SRα cDNA as well as the Eco RI adaptors used for cDNA cloning by inserting them as an Eco RI fragment into the Eco RI site in the polylinker region of pSP64. For expression of SRα in vitro the coding region of SRα was inserted into pSP64T (cut with Bgl II and Bam HI) as a Bam HI fragment excised from pSPSR9 (one Bam HI site comes from the polylinker of pSP64 the other is located in the 3′ untranslated region of SRα at position 2364). In addition to the SRα coding sequence, the resulting plasmid pSPSRα contains the 5′ untranslated region of Xenopus β globin, 22 nucleotides from the polylinker region of pSP64, 15 nucleotides from the Eco RI adaptor used in cDNA cloning, 41 nucleotides of the original 5′ untranslated SRα sequence, and 308 nucleotides of the 3′ untranslated region of SRα under transcriptional control of the SP6 promoter.

The elastase fragment (EF) of SRα starts with a methionine encoded by an ATG at position 495 of the SRα cDNA. This codon was used for initiation of translation of SRα-EF in vitro employing an Ava II site at position 490 of the SRα cDNA. Plasmid pSPSR10 was constructed by excising the SRα coding sequence from pSPSR9 with Eco RI and Bam HI and inserting it into pSP65 cut with Eco RI and Bam HI. After partial digest of this plasmid with Ava II (there are three sites in the SRα cDNA) and Bam HI digest the resulting Ava II–Bam HI fragment was blunt ended using Klenow fragment of polymerase and ligated into the Hind II site in the polylinker region of pGEM-1 to obtain plasmid pSPSR21. To enable transcription in plasmid pSP64T, pSPSR21 was cut with Hind III and Eco RI, and the excised fragment was cloned into pSP64T cut with Bgl II and Bam HI after filling in the ends of both the fragment and vector sequences with Klenow fragment of polymerase. The resulting plasmid, pSPSR-EF, contains the Xenopus β globin 5′ untranslated region, 17 nucleotides carried over from the pGEM-1 polylinker region, 4 nucleotides of SRα cDNA preceding the SRα-EF initiating ATG, 1461 nucleotides encoding SRα-EF, and 308 nucleotides of the SRα 3′ untranslated region under transcriptional control of the SP6 promoter.

Transcription-linked Translation

Transcription of SP6 plasmids was as described previously (Perara and Lingappa, 1985). Aliquots of the transcription reaction mixture were used directly in the translation reactions at a final concentration of 20%. Translation reactions of this kind have been described for reticulocyte lysate (Perara and Lingappa, 1985). Proteins synthesized in vitro were labeled by [^{35}S]methionine included in the reaction and visualized by autoradiography after separation by SDS-PAGE. Protein processing and translocation assays including densitometry were as described (Mize et al., 1986; Andrews et al., 1988). To assay reconstitution of translocation activity, sequential translation reactions were mixed as described in the text.

Postribosomal supernates were prepared by centrifugation at 28 psi for 30 min in the air-fuge after translation was terminated by adding cycloheximide to a final concentration of 100 μM. Unincorporated [^{35}S]methionine was removed by chromatography over 5 vol of Sephadex G25 at 4°C in 10 mM Tris-Ac, pH 7.5, 100 mM potassium acetate, 2 mM magnesium chloride, 1 mM DTT. Products of a 1-ml reticulocyte lysate translation reaction of SRα were affinity purified as described (Tajima et al., 1986) using a 200-μl column of Sepharose 4B containing 1 mg of coupled antibody. The CM Sepharose step was replaced by salt exchange by chromatography on a column containing 5 vol of Sephadex G25 followed by a concentration step using a centricon 30 concentrator (Amicon Corp., Danvers, MA), and 0.01% BSA was added to all buffers in place of the detergent. Elution of SRα from the columns was monitored by liquid scintillation counting.

Cofractionation experiments were performed in sucrose step gradients as described (Gilmore and Blobel, 1985) with the following modifications. The sucrose concentration of the low density step was 0.18 M. The volume of this upper step was always 50 μl. To adjust the ions in the low density step to match those of the high density step, 35 μl of a compensating buffer including 0.25 M sucrose was added to each 20-μl translation reaction. The volume of the high density sucrose cushion (0.5 M) was 100 μl in all assays. The composition of each buffer was based on the physiologic buffer that contained 50 mM Triethanolamine, 150 mM potassium acetate, 2.5 mM magnesium acetate. Alterations from this composition were as indicated; e.g., 2 M urea gradients contained all physiologic ions plus the urea. Microsomes were pelleted by centrifugation for 15 min at 30 psi (~180,000 *g*) (urea and high salt gradients) or 10 min at 20 psi (~110,000 *g*) (physiologic gradients) in an air-fuge with the A-100/30 rotor (Beckman Instruments, Inc.). Gradients were fractionated into two 75-μl aliquots, referred to here as top and middle fractions. The bottom fraction was obtained by adding 75 μl of 1% SDS, 10 mM Tris, pH 9.0, to the tubes and incubating at 60°C for 5 min to solubilize pellets. A second similar treatment, in control experiments, confirmed complete solubilization under these conditions.

For cofractionation experiments liposomes were used essentially as described (Doms et al., 1985). After 90 min synthesis at 24°C reticulocyte lysate translation reactions were terminated by chilling on ice, postribosomal supernates were prepared by centrifugation, and unincorporated [^{35}S]methionine removed by gel filtration as described above. These translation products were mixed with liposomes and/or microsomes and incubated at 24°C for 20 min. The reactions were returned to 4°C and adjusted to 1.3 M sucrose in a final volume of 100 μl by adding a precooled solution of 2 M sucrose. Final ion concentrations in both steps were the same as for translation reactions: 10 mM Tris acetate, pH 7.5, 100 mM potassium acetate, 2 mM magnesium chloride, 1 mM DTT. The heavy sucrose step was overlaid with 100 μl of a similar solution containing 0.25 M sucrose. Centrifugation in the air-fuge with the A-100/30 rotor was for 2 h at 30 psi (~180,000 *g*). These gradients were fractionated into five fractions 50 μl each. The final fraction being the pellet solubilized after 5 min incubation at 60°C in 50 μl of 1% SDS, 10 mM Tris-Ac, pH 9. Aliquots of these fractions were analyzed by SDS-PAGE (5 μl) and scintillation counting (10 μl).

Liposomes were prepared as described previously (Doms et al., 1985) except 0.75% [^{14}C]cholesterol was included as a tracer. The ratio of lipids used was 8:7:2:1 (phosphatidyl ethanolamine/phosphatidyl choline/cholesterol/phosphatidyl serine). The final preparation was centrifuged for 10 s in a microfuge to remove aggregates immediately before use.

Results

To examine the biogenesis of the SRP receptor we analyzed the mechanism of targeting and membrane association for SRα. The strategy employed uses SRα molecules synthesized in cell-free translation reactions to assay interaction with the ER membrane in vitro. Two plasmids were constructed to permit cell-free transcription using the SP6 promoter. One plasmid called pSPSRα encodes the full length molecule SRα and the other, pSPSRα-EF, encodes a deletion mutant composed of amino acids 152–638. This deletion mutant, termed SRα–EF, corresponds to the fragment of the molecule released from microsomes by digestion with elastase (Lauffer et al., 1985). After synthesis in reticulocyte lysate a single major band of the expected molecular weight is generated by each plasmid which, in control experiments, proved immunoprecipitable with a monoclonal antibody (Tajima et al., 1986) directed against the authentic receptor (data not shown).

We used two criteria to establish an in vitro targeting assay. First SRα molecules had to become associated with microsomes in a manner indistinguishable from the endogenous receptor. Second, association with microsomes must be functional; i.e., targeted SRα cell-free translation products should interact with secretory protein nascent chain–ribosome complexes to permit translocation across the microsomal membrane. To measure membrane association of newly synthesized SRα we used a strategy similar to one used previously to displace membrane-bound secretory protein translation complexes from microsomes (Gilmore and Blobel, 1985). In our use of this assay, translation reactions synthesizing either SRα or SRα–EF were supplemented with microsomes, incubated at 24°C, and then aqueous perturbants were added and the microsomes were separated from the reaction mixture by centrifugation over a 0.5 M sucrose step gradient. A variety of aqueous perturbants were employed including salts (NaCl or KoAc), EDTA, and urea. Control experiments indicated that the most stringent conditions compatible with maintaining the integrity of the

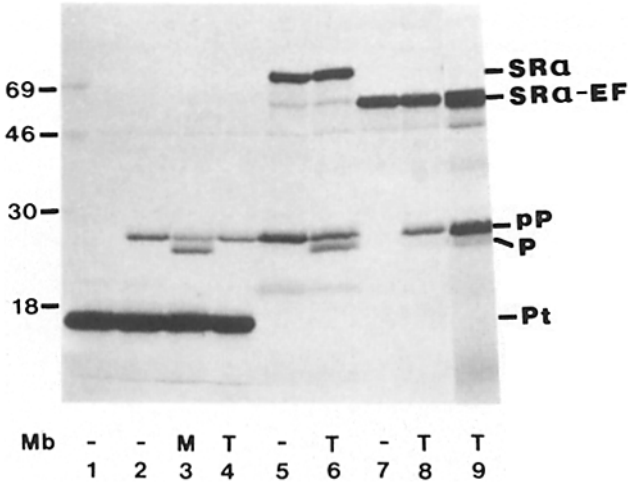

Figure 2. Preincubation with SRα restores translocation activity to trypsinized microsomes. Microsomes were preincubated in 20 μl of translation mixtures synthesizing the control molecule *Pt* (lanes *1–4*) with SRα (lanes *5* and *6*) or SRα–EF (lanes *7–9*). Microsome type is indicated by *Mb*: *M*, untreated microsomes; *T*, trypsinized microsomes; −, no membranes. Subsequent to the preincubation, step reactions were supplemented with a 3-μl aliquot of a newly assembled preprolactin translation reaction (lanes *2–6*, *8*, and *9*). Lane *9* is a longer exposure of lane *8*. Translation products were visualized by autoradiography after separation by SDS-PAGE. Migration positions of molecular mass markers, in kilodaltons, and of the different translation products, including preprolactin (*pP*) and mature translocated prolactin (*P*), are indicated on the sides of the figure.

microsomal membrane, as judged by ability to translocate prolactin, was pelleting in 2 M urea (data not shown).

Localization of SRα by this assay is illustrated in Fig. 1. In the absence of added microsomes (Fig. 1, lanes *1–3*) in reticulocyte lysate translation reactions, newly synthesized SRα is localized almost exclusively at the top of 2 M urea step gradients. When microsomes are added to the reaction cotranslationally, (lanes *4–6*) the sedimentation pattern changes and a substantial fraction of SRα molecules are now found in the pellet at the bottom of the gradients, a location consistent with microsome binding. Under the same conditions SRα–EF does not cofractionate with added microsomes (Fig. 1, compare lanes *7–9* with *10–12*). Therefore resistance to urea extraction is diagnostic for tight association with microsomal membranes. In the SRα translation reactions in Fig. 1 and below there is an additional band present that comigrates with SRα–EF. This product is immunoreactive with antisera directed against SRα and is presumed to result either from internal initiation at the AUG encoding the methionine at the amino terminus of the elastase fragment (Lauffer et al., 1985) or from proteolytic degradation of SRα at the same location. SRα molecules isolated from canine microsomes are very susceptible to proteolytic cleavage at this site (unpublished observations). Regardless of the source of this band, in the pelleting assays in Fig. 1 it behaves the same as SRα–EF.

To demonstrate functional interaction with microsomal membranes it was necessary to first selectively inactivate the endogenous receptor molecules on the cytoplasmic surface of vesicles by digestion with trypsin. Previously trypsinization has been shown to disable translocation by cleaving SRα from microsomes (Gilmore et al., 1982*a*). Mild trypsinization does not inactivate other required components of the translocation machinery as these membranes can be restored by adding back the cytoplasmic portion of SRα (Gilmore et al., 1982*a*).

The translocation properties of trypsinized microsomes after incubation with cell-free translation products of SRα or SRα–EF are shown in Fig. 2. As a mock control for reconstitution we used a molecule composed of amino acids 58–199 of prolactin, referred to here as *Pt*. This molecule lacks a signal sequence and therefore does not interact with microsomes (Andrews et al., 1988). The migration position of Pt in SDS-PAGE is shown in lane *1* of Fig. 2. Reconstitution of translocation function was determined as follows: Trypsinized microsomes were added to reticulocyte translation reactions at the onset of synthesis to permit the test molecules SRα, SRα–EF, or Pt to interact with the membranes cotranslationally. Translation was allowed to proceed for 40 min at 24°C and these microsomes (now referred to as preincubated) were assayed for regained ability to translocate preprolactin. Empirically it was found that sufficient preprolactin synthesis and the most consistent results were obtained if a single new translation reaction (30 μl) was assembled, programmed with RNA encoding preprolactin, and then subdivided into 3-μl aliquots which were added to each of the 20 μl reconstitution reactions. After an additional 40 min of protein synthesis, translation in the combined reactions was terminated by cooling to 0°C, and samples were prepared for electrophoresis. As expected when the original translation reaction contained intact microsomes, some of the preprolactin molecules synthesized were translocated as judged by signal processing to prolactin (compare lane *2* with *3*). Moreover, translation reactions containing trypsinized membranes (lane *4*) do not support preprolactin translocation when preincubated in translation reactions synthesizing the control molecule Pt. However, trypsinized membranes are functionally restored when preincubated with full length SRα molecules and preprolactin is efficiently processed to prolactin (lanes *5* and *6*). SRα–EF is also able to restore some function to trypsinized microsomes, as expected from previous work (Gilmore et al., 1982*b*) (lanes *7–9*).

Preincubation using SRα–EF molecules synthesized in vitro is much less efficient than with the full length molecule (compare lane *6* with *9*; lane *9* is a longer exposure of lane *8*). Densitometry of the autoradiogram in Fig. 2 permitted quantification of the relative translocation activity of trypsinized microsomes preincubated with SRα and with SRα–EF. These molecules restored 78 and 14%, respectively, of the translocation activity observed with an equivalent amount of untrypsinized membranes. No processing of preprolactin was observed with the control molecule Pt after overexposure of lane *4* equivalent to lane *9*. Thus both SRα and SRα–EF molecules synthesized in reticulocyte lysate can associate functionally with microsomes in vitro.

We were surprised that the SRα molecules synthesized in 20 μl reticulocyte lysate translation reactions could restore almost complete translocation activity to trypsinized membranes. Therefore, we measured the amount of SRα synthesized in these reactions to compare with the number of endogenous receptors determined previously (Tajima et al.,

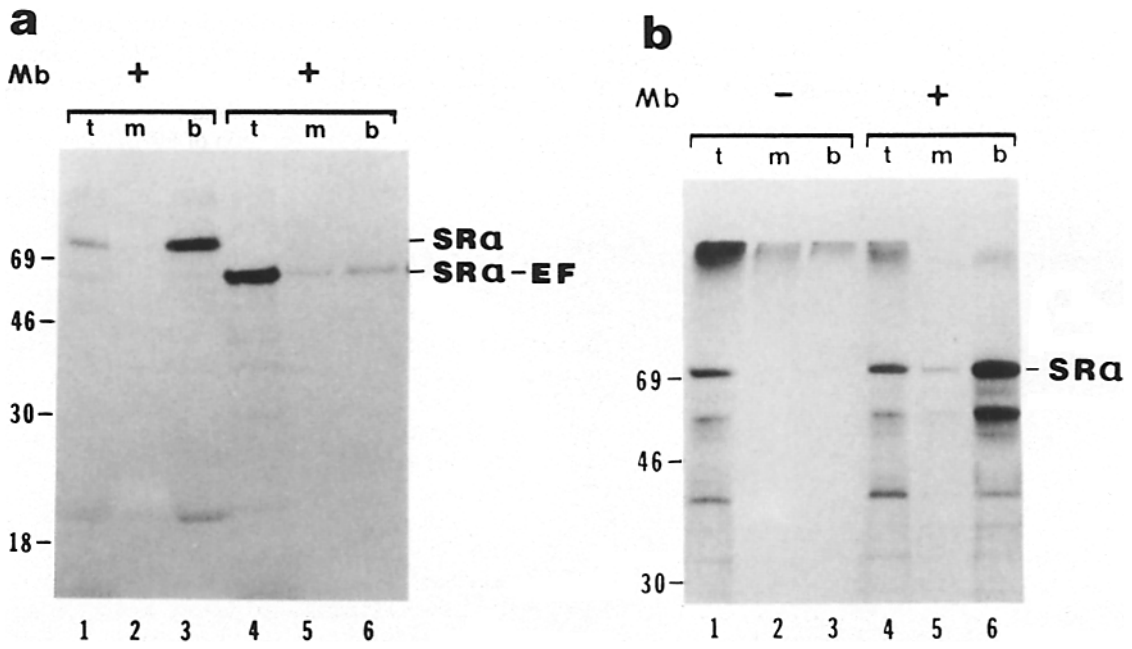

Figure 3. SRα cofractionates with microsomes when added posttranslationally. (*a*) Reticulocyte lysate translation products of SRα (lanes *1–3*) and SRα–EF (lanes *4–6*), desalted by chromatography on Sephadex G25. (*b*) Translation products of SRα purified by affinity chromatography supplemented with microsomes as indicated by *Mb*. Reactions were incubated at 24°C for 20 min, adjusted to 2 M urea, layered on sucrose step gradients, and centrifuged for 15 min at 30 psi. Gradients were divided into top, middle, and bottom fractions, indicated above the lanes as *t*, *m*, and *b*, respectively. Migration positions of molecular mass markers, in kilodaltons, and of SRα and SRα–EF are indicated on the sides of the figure. The bands that migrate faster than SRα in *b* are presumed to result from degradation of intact molecules during purification.

1986). To measure SRα molecules cell-free translation products were labeled during synthesis with ^{3}H-leucine and compared, by densitometry of autoradiograms of SDS-PAGE gels, with a prolactin standard labeled in exactly the same manner. This standard was assayed previously by two independent methods, radioimmune assay and quantitative western blotting (data not shown). We estimate that the amount of SRα synthesized in a 20-μl reticulocyte lysate translation reaction varies between 1 and 20 fmol.

We routinely used 20-μl translation reactions to restore translocation activity to one equivalent of microsomes (defined in Walter and Blobel, 1983). The amount of endogenous SRα in canine microsomes is ~10-fold higher, ~90 fmol/equivalent (Tajima et al., 1986). Therefore 10% of wild-type levels of SRα synthesized in vitro can reconstitute 78% of translocation function. This suggests these molecules are assembled on the membrane in a conformation and at locations appropriate for efficient interaction with the translocation machinery. Moreover since SRα molecules are targeted to trypsinized microsomes, the targeting pathway must differ from the conventional, trypsin-sensitive, SRP-mediated pathway used by secretory and integral membrane proteins.

The targeting assays in Figs. 1 and 2 were performed with microsomes present during synthesis of SRα and SRα–EF. To determine whether or not membrane association was strictly a cotranslational event we used the cell-free targeting assay described above to assay posttranslational association of SRα and SRα–EF with untreated microsomes. To assay SRα targeting posttranslationally, cycloheximide was added to terminate translation and postribosomal supernates were prepared from these reactions by centrifugation. Microsomes were added at 1 eq/20 μl postribosomal supernate and then incubated at 24°C for 20 min, and membrane association was assayed by pelleting in 2 M urea step gradients. In initial experiments, SRα molecules were observed to cofractionate with microsomes (data not shown). Similar targeting was observed for SRα molecules from postribosomal supernates after gel filtration chromatography on G25 Sepharose (Fig. 3 *a*, lanes *1–3*), suggesting small molecules in reticulocyte lysate translations are not required for membrane targeting. As expected, molecules of SRα–EF do not cofractionate with microsomes in 2 M urea step gradients (Fig. 3 *a*, lanes *4–6*). This result is confirmed by our observation that immunopurified SRα also pellets in these gradients only when microsomes are included (Fig. 3 *b*). SRα molecules immunopurified without detergent migrate anomalously in SDS-PAGE (Fig. 3 *b*, lanes *1–3*) and are found near the top of the resolving gel. However when microsomes are added to this same material the migration position returns to normal and SRα molecules cofractionate with microsomes (Fig. 3 *b*, lanes *4–6*). Although other explanations are possible, without added microsomes the behavior of SRα molecules suggests these molecules aggregate after boiling in SDS. Nevertheless without added membranes SRα molecules remain at the top of 2 M urea step gradients (Fig. 3 *b*, lanes *1–3*). When microsomes are added to purified SRα molecules, aggregation is markedly reduced, perhaps due to appropriate targeting of the molecules. We cannot conclude that targeting is independent of all reticulocyte lysate components because a small number of proteins were observed to copurify with SRα on the affinity column (data not shown).

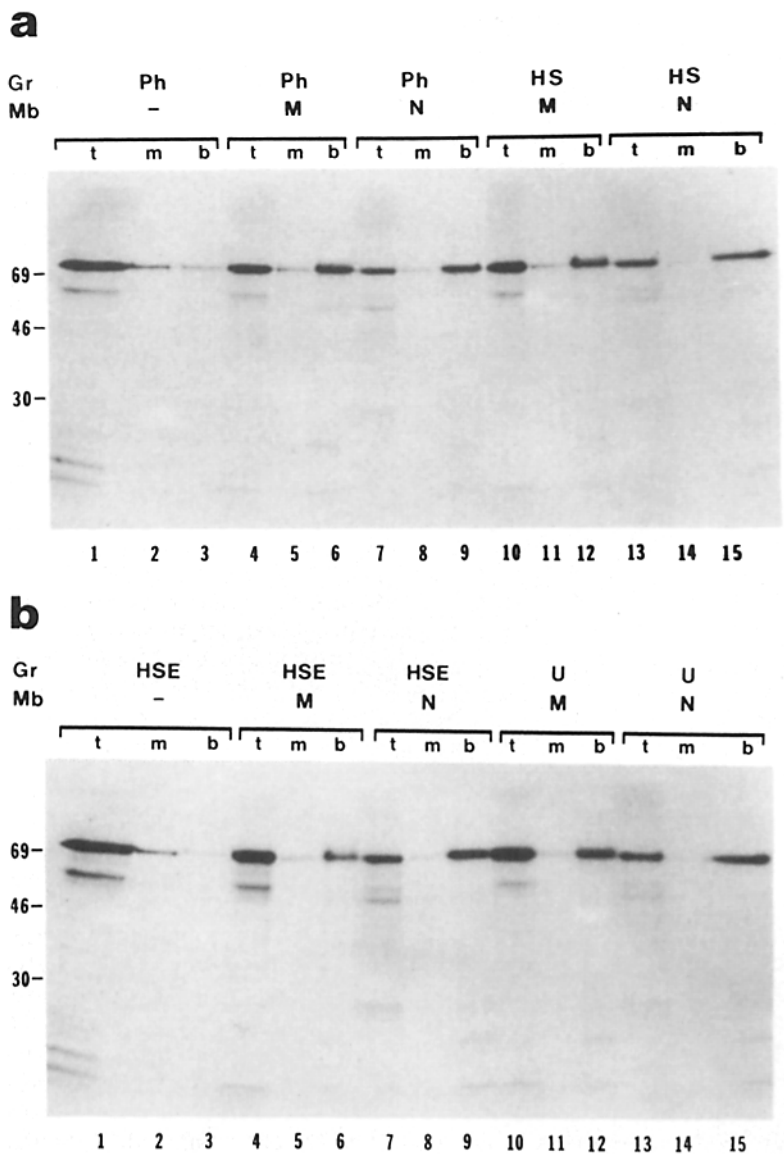

Figure 4. SRα cofractionates with NEM-treated microsomes. Reticulocyte lysate translation reactions of SRα supplemented with microsomes indicated by *Mb*: *M*, untreated microsomes; *N*, microsomes alkylated with NEM; −, no membranes; and analyzed by sedimentation in sucrose step gradients. Gradient conditions (*Gr*) are indicated as: *Ph*, physiologic ions; *HS*, 0.5 M NaCl; *HSE*, 0.5 M NaCl and 25 mM EDTA; *U*, 2 M urea. After centrifugation (as in Fig. 3), gradients were divided into top, middle, and bottom fractions, indicated beneath the bars as *t*, *m*, and *b*, respectively. Positions of molecular mass markers, in kilodaltons, are indicated at the side of the figure. The band below SRα which remains at the top of the step gradients is presumed to result from initiation of translation at an internal methionine.

To assess the possible role of free sulfhydryl groups on the microsome surface for targeting SRα molecules, microsomes were treated with 2 mM NEM and assayed both by pelleting in step gradients and by reconstitution of translocation activity. NEM has been shown to abolish SRP-dependent microsomal membrane translocation activity by alkylating the SRα molecules on the membrane surface thereby disrupting SRP recognition of the receptor (Gilmore et al., 1982*a*). NEM has also been suggested to block a later stage in SRP-mediated translocation (Hortsch et al., 1986). Moreover, modification with NEM also disables the SRP, SRP receptor–independent translocation process described for several polypeptides such as prepromellitin (Muller and Zimmerman, 1987).

In contrast to these processes targeting of SRα synthesized in vitro is not affected by pretreatment with NEM (Fig. 4) as assayed by pelleting in the presence of a variety of aqueous perturbants. The efficiency of SRα targeting to both mock NEM- (*M*) and NEM- (*N*) treated microsomes is identical in step gradients containing physiologic ions (Fig. 4 *a*, lanes *1–9*), 0.5 M NaCl (Fig. 4 *a*, lanes *10–15*), 0.5 M NaCl, 25 mM EDTA (Fig. 4 *b*, lanes *1–9*), and 2 M urea (Fig. 4 *b*, lanes *10–15*).

To determine whether or not this apparent targeting to NEM-treated microsomes results in a functional association with the membrane, translocation activity was measured for microsomes treated with NEM alone or in combination with trypsin. The rationale for digesting microsomes with trypsin before alkylation with NEM was to expose potential alkylation-sensitive sites on the ER membrane masked by trypsin-sensitive proteins including endogenous SRα. In contrast, trypsinization after NEM treatment might expose additional potentially functional sites on the ER membrane otherwise occupied by endogenous SRα.

Translocation activity is restored to microsomes alkylated with NEM by SRα molecules synthesized in reticulocyte lysate (Fig. 5). In this experiment cell-free translation products of plasmids encoding either SRα or, as a control, chimpanzee α globin were supplemented with microsomes after 40 min synthesis at 24°C. After an additional 20 min at this tem-

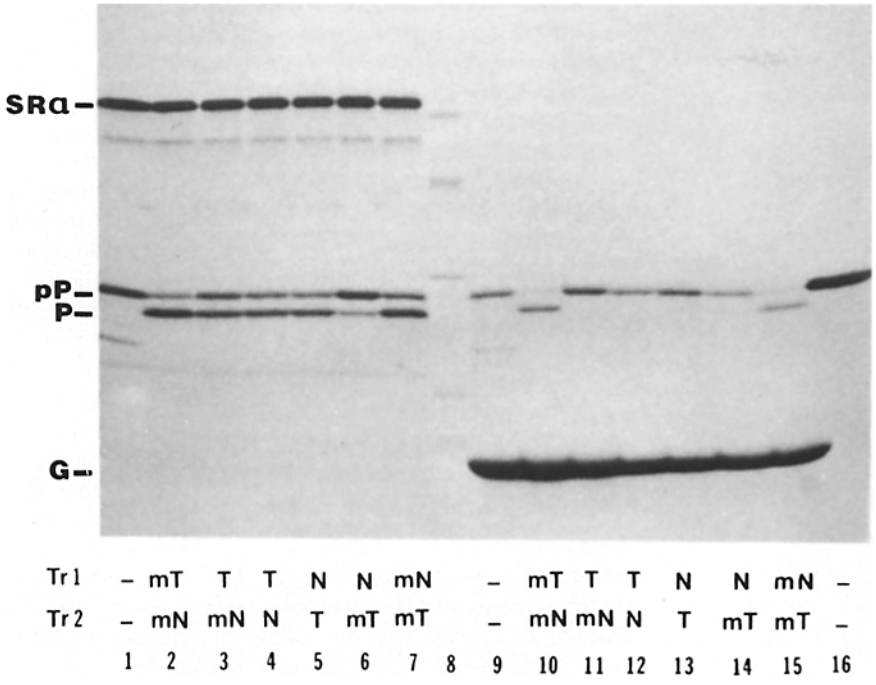

Figure 5. Incubation of microsomes, inactivated by trypsinization and/or alkylation, with SRα restores translocation activity. Microsomes were incubated with SRα, (lanes *1–7*) or Globin (lanes *9–16*) and then supplemented with a newly assembled preprolactin translation reaction. The microsomes added were pretreated in two sequential steps, *Tr1* and *Tr2*, respectively. Microsome treatments: *T*, trypsinized microsomes; *N*, NEM-alkylated microsomes; −, no membranes. The prefix (*m*) indicates mock treatment. Molecular mass markers, lane *8*, are albumin, 69 kD; ovalbumin, 46 kD; carbonic anhydrase, 30 kD; and lactoglobulin, 18 kD. The migration positions of the translation products (*SRα*), preprolactin (*pP*), mature prolactin (*P*), and globin (*G*) are indicated at the side of the figure.

perature these reactions were supplemented with 3 μl of a newly assembled preprolactin translation reaction as above and incubation was continued for a final 40 min. The position of unprocessed preprolactin molecules, SRα, and globin in autoradiograms of these translation reactions after SDS-PAGE are shown in Fig. 5, lanes *1*, *9*, and *16*. When these translation reactions are supplemented with mock trypsinized, mock NEM-treated microsomes (lanes *2*, *7*, *10*, and *15*), preprolactin molecules are translocated and cleaved to mature prolactin, as expected.

Incubating microsomes with SRα molecules but not with globin molecules restores translocation activity abolished by pretreatment with either trypsin or NEM, alone and in combination (compare lanes *3–6* with *11–14*). In almost all cases translocation activity is restored to close to wild-type levels. The one exception is membranes treated only with NEM (lane *6*). In this experiment and others (Fig. 6 *b* and unpublished observations) translocation activity of microsomes treated only with NEM was restored to a lesser extent, discussed below.

If a step in the translocation pathway subsequent to signal cleavage was abolished by either trypsin or NEM, signal processing might occur without molecules actually being translocated. Furthermore, if SRα molecules, synthesized in vitro, caused microsomes to become destabilized then signal peptidase activity might be released from the membranes. In either case preprolactin molecules would be cleaved to prolactin without being transported to the ER lumen. To distinguish fully translocated prolactin molecules from those processed but incompletely or not translocated, we assayed for protection from proteinase K. After adjusting translation reactions to 0.1 mg/ml proteinase K for 45 min at 0°C the enzyme was inactivated by boiling in SDS and samples were immunoprecipitated with anti-prolactin antiserum. Immunoprecipitation removes from the analysis protease resistant degradation products of SRα which might otherwise complicate interpretation of the autoradiograms.

Protease protection assays for trypsinized microsomes repopulated with SRα are presented in Fig. 6 *a*. Preprolactin, (Fig. 6 *a*, lane *1*) is sensitive to protease (Fig. 6 *a*, lane *2*). However, preprolactin molecules processed to prolactin in the presence of SRα reconstituted trypsinized microsomes (lane *3*) are protected from protease (lane *4*), unless the microsomal membrane is solubilized with nonionic detergent (lane *5*). In this assay mock trypsinized membranes process and translocate prolactin with similar efficiency (lanes *6–8*). Microsomes inactivated by alkylation with NEM and subsequently reconstituted with SRα also regain authentic translocation activity (Fig. 6 *b*, lanes *1–5*). Although reconstituted NEM-treated microsomes do not regain translocation efficiency equal to mock-treated reconstituted microsomes (compare lanes *3* and *4* with *6* and *7*), all of the molecules with cleaved signal sequences are protected from protease. As expected in reactions in which NEM-treated microsomes were mock reconstituted with α globin molecules, preprolactin processing is not observed (lane *9*, nor are any prolactin immunoreactive species observed to be protected from protease; lanes *10* and *11*). Therefore, during the manipulations of these assays, microsomes are not being disrupted such that signal peptidase can act on untranslocated molecules. Moreover since incubation with newly synthesized SRα efficiently reconstitutes the translocation pathway in microsomes, later steps in the pathway are not being affected by the NEM treatment used here.

These experiments strongly suggest the SRα molecules are not targeted to microsomal membranes by the conventional SRP-SRP receptor-mediated translocation pathway. However, they do not address the actual mechanism of targeting. One difference between SRα and SRα-EF is the presence of two relatively hydrophobic stretches of amino

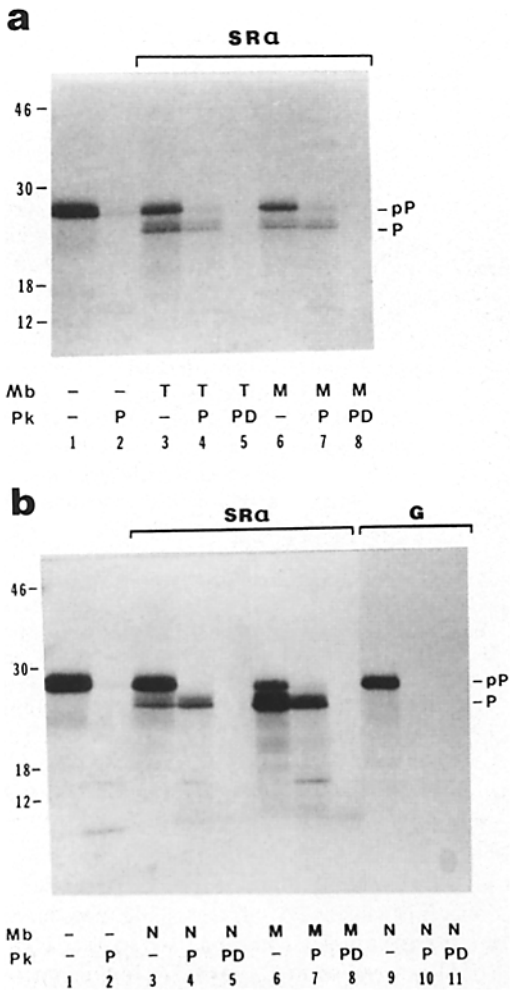

Figure 6. Localization of translocated prolactin molecules by protease protection assays. Microsomes were incubated with SRα or globin (*G*) after digestion with trypsin (*a*) or alkylation with NEM (*b*). Then preprolactin translation reactions were added to these reactions. Microsomes added to the repopulation reactions (*Mb*) are indicated: −, no membranes; *T*, trypsinized; *N*, NEM alkylated; and *M*, untreated microsomes. After 40 min protease was added (*Pk*) as indicated: *P*, proteinase K (0.1 mg/ml); *PD*, proteinase K plus Triton X-100 detergent (0.1%). To distinguish translocated from nontranslocated molecules proteolysis products were immunoprecipitated with antiserum to prolactin and were visualized by SDS-PAGE and autoradiography. Positions of molecular mass markers, in kilodaltons, and of preprolactin (*pP*) and prolactin (*P*) are indicated at the sides.

acids present in full length molecules. It has been postulated that these sequences act to anchor the receptor in the ER membrane. Therefore, the targeting event we are assaying could be due to simple partitioning of one or both of these sequences in the lipid bilayer of the microsome. Such a targeting event would be analogous to the insertion sequence–mediated membrane association of cytochrome b5 (Anderson et al., 1983). If a relatively nonspecific hydrophobic interaction is responsible for the cofractionation observed with microsomes then molecules of SRα would be expected to interact similarly with artificial phospholipid vesicles.

To examine the specificity of SRα targeting we assayed binding to liposomes by centrifugation in sucrose step gradients (Doms et al., 1985). In these experiments liposomes can be easily distinguished from microsomes because these vesicles rise to the top of sucrose step gradients while microsomes pellet during centrifugation. Liposome movement in step gradients was monitored by including a small amount, 0.75%, [^{14}C]cholesterol during liposome preparation. As expected almost all of the radioactivity is recovered from the low density sucrose step (fractions *1* and *2*) when liposomes are mixed with a mock SRα translation reaction, adjusted to 1.3 M sucrose, overlaid with a 0.25 M sucrose solution, and centrifuged at 30 psi for 2 h. The percent of total radioactivity in each fraction of one such gradient, measured by scintillation counting, is presented in Fig. 7 *a*. The bottom fraction, number 5, represents pelleted material.

Postribosomal supernates of reticulocyte lysate translation reactions of SRα were salt exchanged on a Sephadex G25 column to remove unincorporated [^{35}S]methionine which would interfere with scintillation counting to localize liposomes. This material was then added to microsomes, liposomes, or a mixture of microsomes and liposomes, and centrifuged in parallel with the liposome gradient described above (Fig. 7 *a*). When microsomes were added to preparations of SRα, targeted molecules were recovered from the bottom fraction of step gradients as expected (Fig. 7 *b*, fractions *1–5*). In this gradient SRα molecules were not observed in fractions corresponding to the low density step, fractions *1* and *2*. When the same analysis is performed with liposomes (Fig. 7 *c*), SRα molecules are found only in their original location, the heavy sucrose step (fractions *3* and *4*), and do not comigrate with liposomes to the lighter fractions (*1* and *2*), or pellet on the bottom of the tube (fraction *5*). When liposomes and microsomes were mixed before centrifugation, a proportion of SRα molecules are again observed to cofractionate with microsomes but not liposomes (Fig. 7 *d*). The migration of liposomes from the high density sucrose step to the low density sucrose step in gradients containing both liposomes and microsomes (Fig. 7 *d*) was essentially the same as that shown for liposomes alone in *a* (data not shown).

To examine the vesicle specificity of the targeting reaction, a mitochondrial-enriched fraction was prepared from canine pancreas by differential centrifugation. Electron micrographs (not shown) confirmed that the preparation contained primarily mitochondria. This mitochondria-enriched fraction was used in place of microsomes, at a total protein content four times higher than the minimum required to detect targeting to microsomes, in the 2 M urea pelleting assay described above. Targeting to this fraction is not observed as the distribution of SRα molecules in these gradients is indistinguishable from control gradients (Fig. 8, compare lanes *1–3* with *4–6*).

The interaction between SRα molecules synthesized in vitro and microsomes was examined in greater detail by varying the relative amount of microsomes and translation products in the reactions before cofractionation in 2 M urea gradients. Increasing the amount of microsomes added to reactions cotranslationally increases the proportion of molecules that become tightly associated with microsomes and are therefore recovered in pellets after centrifugation (Fig. 9 *a*). After an initial rapid increase in the proportion of molecules that become microsome associated, binding saturates

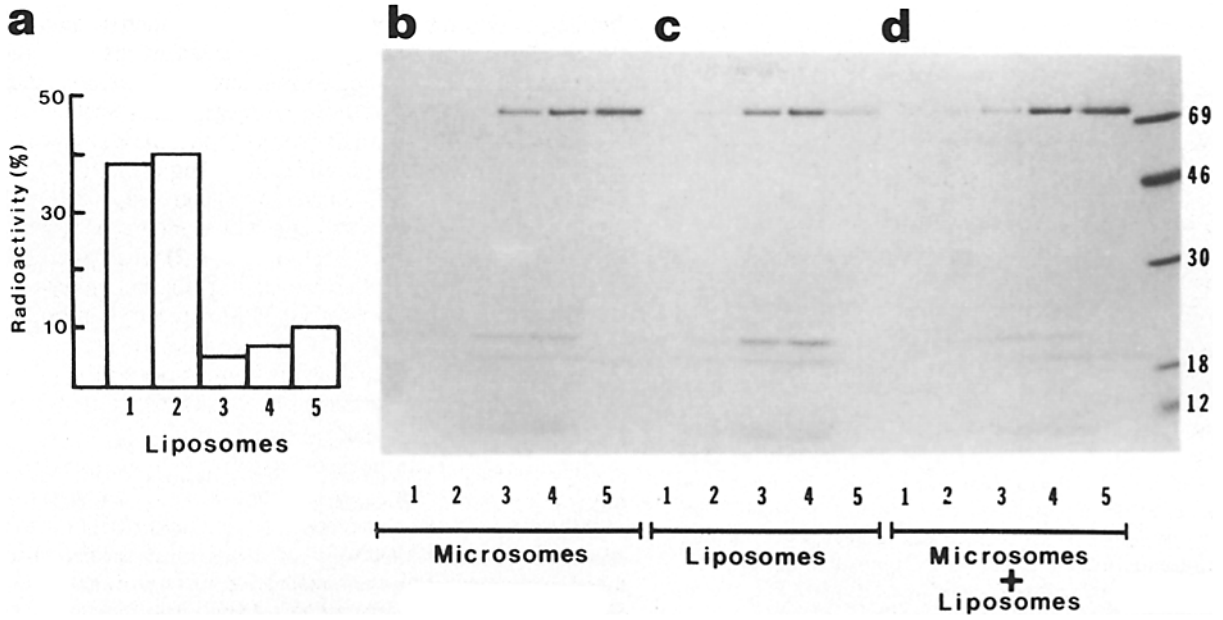

Figure 7. SRα cofractionates with microsomes but not liposomes in sucrose step gradients. (*a*) Liposomes containing 0.75% [^{14}C]cholesterol were adjusted to 1.3 M sucrose in a final volume of 100 μl and overlaid with 100 μl of a 0.25 M sucrose solution. After centrifugation at 30 psi for 2 h in an air-fuge (Beckman Instruments, Inc.), gradients were divided into five 50-μl fractions. The bottom fraction, number 5, represents any pelleted material solubilized in 50 μl of 1% SDS, 10 mM Tris, pH 9, after incubation at 60°C for 5 min. The radioactivity of each fraction was measured by scintillation counting and plotted as percent of the total. (*b–d*) Reticulocyte lysate translation products of SRα were desalted on Sephadex G25 and mixed with microsomes, liposomes, or microsomes plus liposomes, as indicated and subjected to centrifugation as in *a*. Aliquots of each fraction corresponding to 0.5 μl of SRα translation products were separated by SDS-PAGE and visualized by autoradiography to localize SRα molecules. The migration position of molecular mass standards is indicated at the side of the autoradiogram. Liposomes were localized by measuring total radioactivity in similar aliquots by scintillation counting.

at about four equivalents of microsomes in a 20-μl translation reaction. This pattern of binding suggested that 20 μl of translation products (~10 fmol SRα) could saturate the unused binding sites on approximately two to three equivalents of microsomes.

To demonstrate saturation of binding sites directly we held the concentration of microsomes constant and varied the amount of SRα molecules added to reactions. Post ribosomal supernates of SRα translation reactions were prepared and salt exchanged as described above. The specified volume of this material was added to one equivalent of microsomes after the volume of the reaction was adjusted to 20 μl with buffer (10 mM Tris, pH 7.5, 100 mM potassium acetate, 2 mM magnesium chloride, 1 mM DTT) and then incubated at 24°C for 20 min. As expected when larger amounts of SRα translation products are added to a fixed concentration of microsomes the proportion of the total SRα recovered from the material pelleted in 2 M urea sucrose step gradients is reduced (Fig. 9 *b*). The shape of the curve is consistent with saturation of one equivalent of microsomes by ~10 μl of SRα translation products (~5 fmol of SRα).

These results suggest that there is a specific targeting site for SRα on the microsomal membrane. Furthermore, SRα binding experiments employing trypsinized microsomes suggest that resistance to extraction by 2 M urea is protein mediated (Fig. 10). Although molecules of SRα are able to restore translocation function to trypsinized microsomes (Figs. 2 and 5) this association is not as stable as with untrypsinized or NEM-treated microsomes. As shown above, SRα binding to mock-treated microsomes and NEM-treated microsomes (Figs. 1, 3, and 4) results in an association with the microsome stable to 2 M urea. However, when SRα-reconstituted trypsinized microsomes are assayed as above using 2 M urea step gradients, SRα molecules are released (Fig. 10 *a*, compare lanes *1–3* with *4–6*). Nevertheless, SRα molecules are interacting with microsomes because these molecules do cofractionate with microsomes in step gradients containing physiologic ions (lanes *7–12*). Control experiments such as that shown above in Fig. 4 demonstrate that SRα molecules do not pellet in physiologic gradients without added microsomes.

Moreover, when trypsinized microsomes reconstituted with SRα are pelleted in sucrose step gradients under physiologic conditions and resuspended in a preprolactin translation reaction they are active in promoting translocation (Fig. 10 *b*). As a control mock trypsinized microsomes were resuspended in a prolactin translation reaction after a similar incubation. As expected resuspended pellets of intact microsomes are active in translocating preprolactin (lanes *1–3*). Furthermore, the small number of SRα molecules that remain associated with trypsinized microsomes after pelleting also function to translocate prolactin (lanes *4–6*). Trypsinized microsomes do not translocate prolactin without the reconstitution step as shown in lane *7*. Together these results suggest that the targeting site for SRα is trypsin resistant while stable membrane anchoring is mediated by an additional trypsin-sensitive component. Also, stable membrane anchoring is not required for SRα to be active in promoting preprolactin translocation.

The most logical candidate protein to mediate one or both

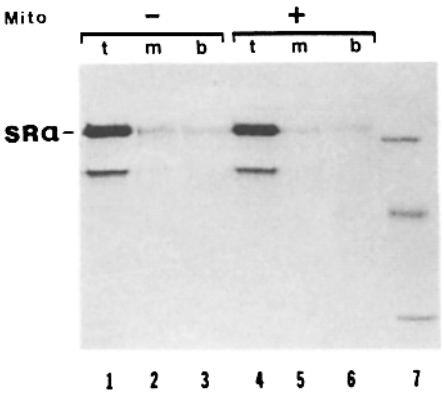

Figure 8. SRα molecules do not cofractionate with mitochondria enriched vesicles. Reticulocyte lysate translation products of SRα were incubated with a vesicle preparation enriched for mitochondria (*Mito*). After the repopulation step, reaction products were separated by centrifugation on sucrose step gradients in 2 M urea as above. Molecular mass markers (lane 7) are albumin (69 kD), ovalbumin (46 kD), and carbonic anhydrase (30 kD). The migration position of SRα is indicated at the side. The band in the autoradiogram below SRα is presumed to originate from initiation of translation at an internal methionine.

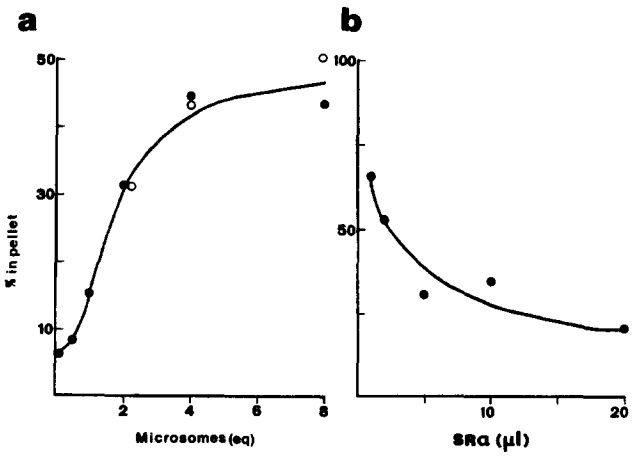

Figure 9. Binding of SRα molecules to microsomes is saturable. (*a*) Microsomes were added cotranslationally, in the amount indicated, to 20-μl SRα reticulocyte lysate translation reactions. (*b*) One equivalent of microsomes was added posttranslationally to the specified volume of a postribosomal supernate prepared from a SRα reticulocyte lysate translation reaction. The volumes of the reactions were normalized to 20 μl by adding the required volume of a mock translation mixture. Binding of SRα molecules to microsomes was assayed by cofractionation in 2 M urea step gradients, as above. The proportion of SRα molecules in each fraction was determined by densitometry of autoradiograms after separation by SDS-PAGE. • and ○ represent two independent experiments.

of these steps in SRα biogenesis is SRβ. SRα and SRβ have been shown to form a complex stable to high salt (Tajima et al., 1986). Furthermore the two molecules have been shown to cofractionate with constant stoichiometry when rough and smooth liver microsomes are separated on sucrose gradients (Tajima et al., 1986). If SRβ plays a role in targeting SRα to the microsome we would expect it to be resistant to low concentrations of trypsin. However, if SRβ alone is responsible for mediating anchoring of SRα on the ER membrane it should be sensitive to digestion with trypsin at concentrations that prevent tight association between full length SRα molecules and microsomes in vitro, ~2 μg/ml.

The sensitivity of both SRα and SRβ molecules to digestion with trypsin was measured by incubating microsomes with different concentrations of the enzyme followed by immunologic identification of the digestion products after electrophoretic separation and blotting on nitrocellulose. As expected molecules of SRα are sensitive to low concentrations of trypsin (Fig. 11, *top section*). Degradation products of this molecule appear after incubation with as little as 1 μg/ml trypsin (lanes *4–6*), and digestion is essentially complete by 5 μg/ml (lanes *7–9*). In contrast, SRβ molecules are much less sensitive to the enzyme as degradation products first appear after incubation with 30 μg/ml trypsin (lanes *10–12*). This pattern of trypsin sensitivity (Fig. 11, *bottom section*) is consistent with a role for SRβ in targeting rather than stable anchoring of SRα molecules on the ER membrane.

Discussion

We have elucidated a previously uncharacterized mechanism for the biogenesis of SRα molecules that can be reconstituted in vitro. This pathway appears to involve at least two distinct steps: first, targeting to specific sites on the ER membrane and second, anchoring of the targeted molecule firmly to the cytoplasmic face of the membrane. The targeting step was assayed as the ability of SRα synthesized in vitro to substitute functionally for endogenous SRα on microsomal membranes inactivated by digestion with trypsin or by alkylation with NEM (Figs. 2, 5, and 6). The membrane-anchoring step was assayed by resistance to extraction with 2 M urea (Figs. 1, 3, and 4).

Targeting of SRα

The initial evidence for a highly selective targeting pathway comes from determination of the number of SRα molecules synthesized in vitro and the efficiency with which they act to restore translocation function to inactivated microsomes. We estimate that no more than 10 fmol of SRα molecules are synthesized in a 20-μl reticulocyte lysate translation reaction. Nevertheless, when these molecules are used to repopulate one equivalent of trypsinized microsomes, ~80% of the original translocation activity is restored (Fig. 2). In contrast, one equivalent of untreated microsomes has been shown to contain ~90 fmol of SRα (Tajima et al., 1986). Therefore, replacing 10% of the endogenous SRP receptor population restores almost 80% of the translocation activity. This result suggests SRα molecules synthesized in vitro are specifically targeted to sites on the ER membrane. Moreover it suggests that either SRP receptor concentration is not the limiting factor in the translocation of prolactin molecules across the ER membrane in vitro or that most of the SRP receptor on the surface of isolated microsomes is not capable of promoting translocation of an added substrate. Endogenous SRP receptor may appear nonfunctional for any of a variety of reasons including denaturation during microsome preparation and masking by other potential substrates or regulatory effectors.

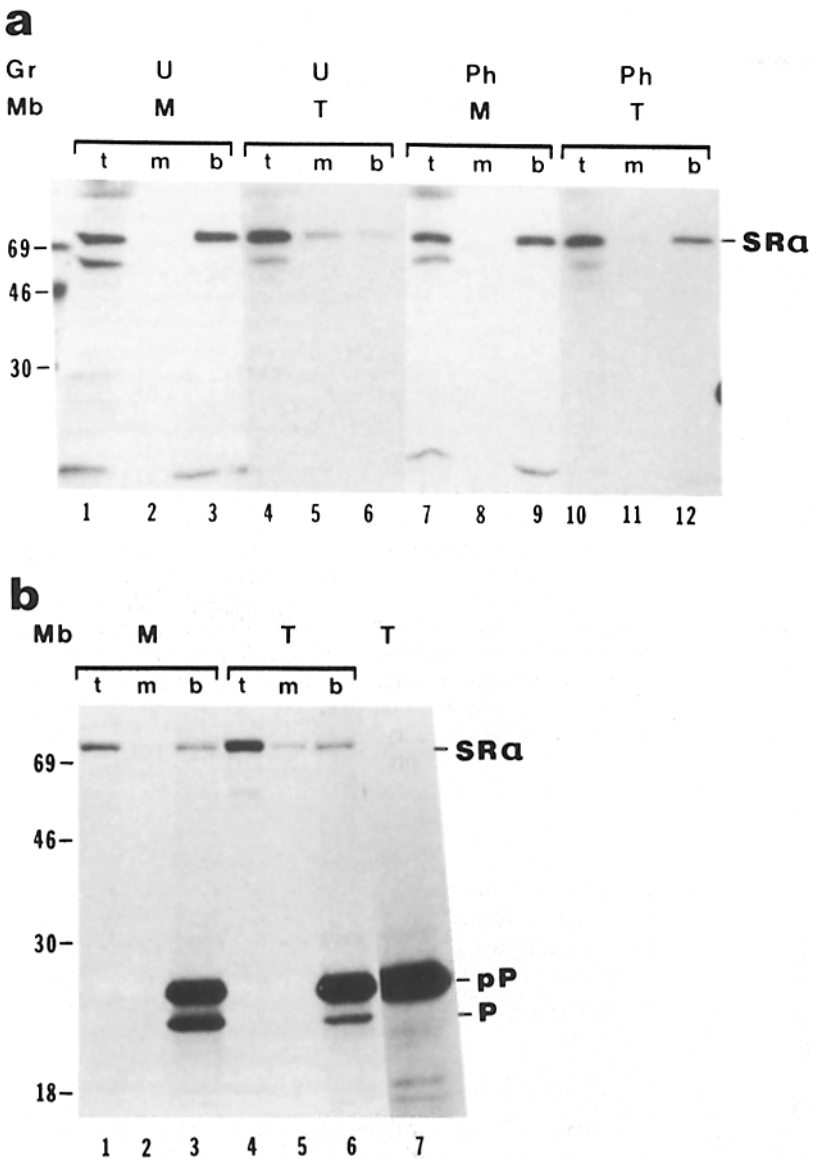

Figure 10. Binding of SRα molecules to trypsinized microsomes is not urea resistant. Microsomal membranes (*Mb*) were added to SRα reticulocyte lysate translation reactions as intact microsomes (*M*) and trypsinized microsomes (*T*). SRα binding to microsomes was assayed in *a* by cofractionation in sucrose step gradients (*Gr*) containing either 2 M urea (*U*) or physiologic ions (*Ph*) as above. In *b* microsomes, indicated as in *a*, were repopulated with SRα in a similar fashion and fractionated over sucrose step gradients in physiologic ions (lanes *1–6*). Translocation activity restored by targeted SRα molecules was measured for microsomes pelleted in these gradients by resuspending the pellets in newly assembled preprolactin reticulocyte lysate translation reactions (lanes *3* and *6*) and determining the extent of processing of preprolactin molecules to prolactin by densitometry of the autoradiograms. As a control, untreated trypsinized microsomes were added to a similar preprolactin translation reaction (lane *7*). The migration positions of the translation products (*SRα*) preprolactin (*pP*), mature prolactin (*P*), and of molecular mass markers, in kilodaltons, are indicated at the sides of the figure.

Reconstitution of translocation function to microsomes inactivated by alkylation with NEM provides some clues as to the specific site the newly synthesized SRα molecules occupy on the ER membrane. Alkylation with NEM is unlikely to displace endogenous SRα from SRβ molecules. Therefore, newly synthesized SRα molecules are probably recruited to unoccupied sites on the membrane. The lower translocation activity of SRα reconstituted NEM-treated microsomes relative to those reconstituted similarly but inactivated with trypsin is consistent with there being a smaller number of potentially active sites on NEM-treated microsomes (Figs. 5 and 6). In contrast, NEM-treated microsomes treated with trypsin either before or after the alkylation step can be restored to the same level of translocation as microsomes treated only with trypsin. These results are consistent with the newly synthesized SRα molecules occupying additional sites exposed by the trypsinization step. Since the endogenous SRα molecules are trypsin sensitive it seems likely that the sites exposed by trypsin were previously occupied by endogenous SRα molecules. For this reason we refer to inactivated microsomes with reconstituted translocation activity due to added SRα molecules as repopulated microsomes.

Further evidence for specific targeting of SRα comes from estimates of the relative number of SRα molecules synthesized compared to the number of prolactin molecules translocated by SRα repopulated microsomes. The relative number of these molecules can be estimated from measured optical densities of bands on autoradiograms by correcting for the number of methionine residues in each molecule. Although the experiments presented in Figs. 2 and 5 can be used for this purpose the experiment presented in Fig. 10 *b* is the most appropriate. In this experiment microsomes were separated from unbound SRα molecules after the repopulation step by pelleting in step gradients. Therefore, when these microsomes were resuspended in a preprolactin translation reaction only those SRα molecules that cofractionated with microsomes could provide access to the translocation machinery. We calculate that the number of prolactin mole-

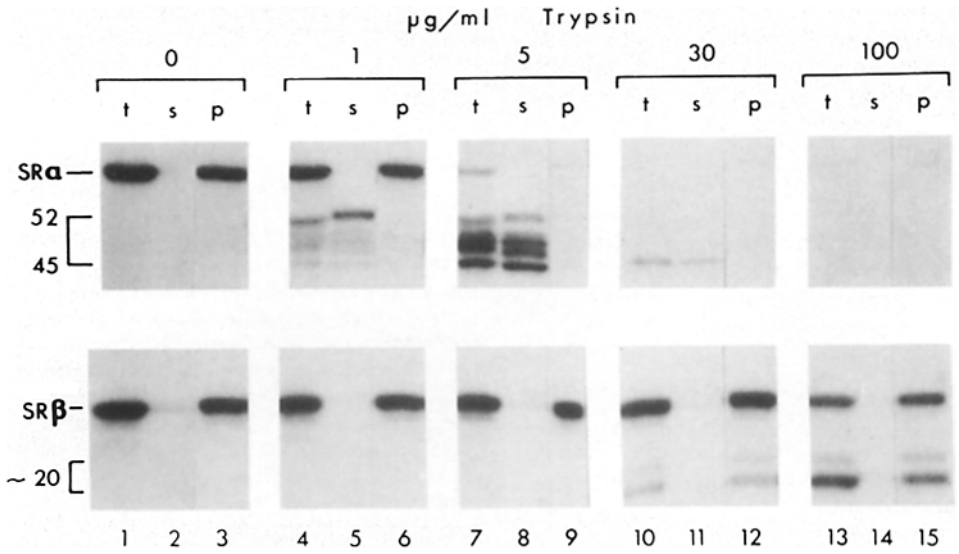

Figure 11. SRβ is not sensitive to concentrations of trypsin which degrade SRα. Microsomes were incubated with trypsin at the concentrations indicated for 1 h at 0°C. The reaction products were divided into two equal aliquots and one was fractionated into a supernate and a microsome-associated pellet by centrifugation. The three fractions representing total products (*t*), products released from microsomes and found in the supernate (*s*), and those which remain bound to microsomes recovered in pellets (*p*) were then separated by SDS-PAGE and electrophoretically transferred to nitrocellulose. SRα and SRβ molecules were identified by probing the nitrocellulose blots with monoclonal antibodies directed against each of the molecules and an anti-mouse IgG second antibody labeled with ^{125}I, followed by autoradiography. Bands identified as SRα and SRβ and the migration positions of molecular mass markers, in kilodaltons, are indicated.

cules translocated in the experiment illustrated in Fig. 10 *b* was sixfold larger than the number of SRα molecules available to initiate translocation. This result strongly suggests a large fraction of the SRα molecules that became associated with microsomes during the repopulation step are active to translocate prolactin molecules.

Targeting of SRα molecules to the ER membrane differs from SRP-mediated targeting of secreted proteins in a number of ways. Targeting is independent of SRP receptor and can take place posttranslationally (Figs. 2, 3, and 4). In addition, there does not appear to be a requirement for small molecules such as nucleoside triphosphates, other than those possibly provided by the extensively washed microsome fraction. Although we have not measured the levels of such molecules directly, a gel filtration step that removes unincorporated [^{35}S]methionine does not reduce targeting efficiency (Fig. 3 *a*). Moreover, targeting is independent of SRP, requiring at most a small subset of proteins in the translation reaction, as SRα molecules synthesized in vitro are still able to target to microsomes even after affinity purification (Fig. 3 *b*).

The fact that SRα is efficiently targeted to NEM-alkylated microsomes differentiates this pathway from the SRP-independent signal-mediated targeting pathway described for prepromellitin (Muller and Zimmermann, 1987). This pathway has been shown to be disabled when microsomes are alkylated with NEM (Zimmerman and Mollay, 1986). If NEM disables a step in this translocation pathway subsequent to targeting, it is possible that SRα targeting might overlap with the initial steps of this SRP-independent pathway. However, such a step cannot be shared with the SRP-dependent translocation pathway reconstituted to NEM-alkylated microsomes by SRα.

The first 151 amino acids of SRα may function to promote efficient targeting as the deletion mutant SRα-EF appears to associate with microsomes with lower fidelity than the full length molecule as judged by reduced ability to restore translocation (Fig. 2). However, the experiments presented here do not permit us to differentiate a direct effect of the amino terminal region of SRα from improper folding of the deletion mutant. Efficient targeting is observed to microsomes digested with trypsin, suggesting the targeting receptor which may recognize the amino terminal region of SRα is protease resistant. Although we have no direct evidence, the resistance of SRβ to digestion with trypsin (Fig. 11) combined with previously reported colocalization experiments (Tajima et al., 1986), suggests this molecule may play a role in SRα targeting.

SRα Membrane Anchoring

Although SRα molecules are clearly targeted to trypsinized microsomes to restore function (Figs. 2, 5, and 10 *b*) these molecules are not anchored tightly to the membrane. Targeted SRα molecules cofractionate with trypsinized microsomes only in physiologic gradients and are largely released from the membrane by extraction with 2 M urea (Fig. 10 *a*). For this reason we suggest targeting and stable membrane association are distinct steps in SRα biogenesis, which can be dissociated by trypsinization of the membrane.

We examined the specificity of the membrane anchoring event to confirm that the acquisition of urea resistance does not result from simple hydrophobic interaction with the lipid

bilayer. Even under physiologic conditions SRα molecules were not observed to interact with either artificial phospholipid vesicles or a mitochondria-enriched fraction used as nonspecific targets for SRα molecules in cell-free repopulation assays (Figs. 7 and 8). This lack of interaction with liposomes distinguishes the SRα anchoring mechanism from the insertion sequence mediated membrane anchoring of cytochrome b5 (Bendzko et al., 1982). Moreover, additional evidence for a specific microsomal protein required for SRα anchoring was provided by our demonstration that microsomal binding is saturable (Fig. 9). The results obtained by varying either the amount of microsomes or SRα were remarkably similar and suggest that the available sites on one equivalent of microsomes can be saturated by <10 fmol of SRα.

Previous studies have implicated the amino terminal 151 amino acids of SRα in membrane anchoring. Those experiments used proteolytic cleavage of endogenous SRα molecules with either elastase or trypsin to remove the amino terminus from the molecule. When this cytoplasmic fragment of SRα was added back to trypsinized microsomes, translocation activity was restored but SRα molecules did not become tightly associated with the membrane (Gilmore et al., 1982*b*; Meyer and Dobberstein, 1980; Hortsch et al., 1985). These observations led to the conclusion that the two hydrophobic stretches deleted from the molecule mediated SRα membrane association (Lauffer et al., 1985).

In view of the evidence discussed above for protein-mediated membrane anchoring and that showing full length SRα molecules do not become stably associated with trypsinized microsomes (Fig. 10), it is possible that release of the cytoplasmic portion of SRα by proteases is due to degradation of an anchoring protein rather than to cleavage of SRα. If this is true stable membrane association could result from interaction of this putative anchoring protein with any portion of SRα. However, our demonstration that SRα but not SRα–EF associates with intact microsomes in a urea-resistant manner confirms that the amino terminus of SRα is required for membrane anchoring (Figs. 1 and 3). Although it remains a formal possibility that the inability of SRα–EF to interact stably with the membrane is due to improper folding of the deletion mutant instead of loss of required sequences. We consider this possibility unlikely because SRα–EF can function to promote translocation, albeit with reduced efficiency (Fig. 2). Nevertheless, the fact that full-length SRα molecules do not associate tightly with trypsinized microsomes strongly suggests some trypsin-sensitive protein is, at least transiently, involved in SRα anchoring.

Alkylation of microsomes with NEM was used to probe the nature of this putative anchor protein. Alkylation does not prevent SRα binding to microsomes in a urea resistant manner (Fig. 4). Moreover, once anchored to microsomes SRα molecules are also unaffected by 0.5 M NaCl (Fig. 4 *a*, lanes *10–15*), 0.05 M KoAc (not shown), 0.5 M NaCl, 25 mM EDTA (Fig. 4 *b* lanes *1–9*), and 2 M urea, 0.5 M NaCl (not shown). These results, together with our demonstration that the amino terminus of SRα, which contains two hydrophobic stretches of amino acids, is required for tight membrane association, suggest the stabilizing interaction may be primarily hydrophobic. If so, this would explain why this putative membrane anchor is not seen in fractionation experiments in which the microsomal membrane is solubilized with detergents (Tajima et al., 1986).

Although such interaction may be mediated solely by SRβ we consider this unlikely because anchoring is abolished by pretreatment of microsomes with concentrations of trypsin which have no apparent effect on SRβ (compare Fig. 10 with Fig. 11). However, it is possible that the putative anchoring protein interacts with SRα only transiently to stabilize association with the microsome. In such a scenario, a structural or chemical modification catalyzed by the putative anchoring protein may attach SRα to either SRβ, some other microsomal component, or with the lipid bilayer. The results described here provide a logical framework and experimental system that will permit such questions to be addressed.

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